



Binding of phospholipids to β -Lactoglobulin and their transfer to lipid bilayers

P.A.T. Martins, F. Gomes, W.L.C. Vaz, M.J. Moreno *

Departamento de Química da FCTUC, Rua Larga, 3004-535 Coimbra, Portugal

ARTICLE INFO

Article history:

Received 6 November 2007

Received in revised form 21 February 2008

Accepted 21 February 2008

Available online 4 March 2008

Keywords:

Phospholipid exchange

Phospholipid binding

β -Lactoglobulin

Protein aggregation

POPC bilayers

Protein adsorption

ABSTRACT

The bovine milk lipocalin, β -Lactoglobulin (β -LG), has been associated with the binding and transport of small hydrophobic and amphiphilic compounds, whereby it is proposed to increase their bioavailability. We have studied the binding of the fluorescent phospholipid-derivative, NBD-didecanoylphosphatidylethanolamine (NBD-diC₁₀PE) to β -LG by following the increase in amphiphile fluorescence upon binding to the protein using established methods. The equilibrium association constant, K_B , was $(1.2 \pm 0.2) \times 10^6 \text{ M}^{-1}$ at 25 °C, pH 7.4 and $I = 0.15 \text{ M}$. Dependence of K_B on pH and on the monomer–dimer equilibrium of β -LG gave insight on the nature of the binding site which is proposed to be the hydrophobic calyx formed by the β -barrel in the protein. The monomer–dimer equilibrium of β -LG was re-assessed using fluorescence anisotropy of Tryptophan. The equilibrium constant for dimerization, K_D , was $(7.0 \pm 1.5) \times 10^5 \text{ M}^{-1}$ at 25 °C, pH 7.4, and 0.15 M ionic strength. The exchange of NBD-diC₁₀PE between β -LG and POPC lipid bilayers was followed by the change in NBD fluorescence. β -LG was shown to be a catalyst of phospholipid exchange between lipid bilayers, the mechanism possibly involving adsorption of the protein at the bilayer surface.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

The bovine milk protein β -Lactoglobulin (β -LG) belongs to the lipocalin protein family and is characterized by a β -barrel of eight anti-parallel β -strands with a hydrophobic interior [1]. Two forms of this protein are usually found, A and B, differing only in two amino acid residues, Asp 64 Gly and Val 118 Ala [2]. The physiological role of β -LG is uncertain but, because it binds small hydrophobic molecules (e.g. retinol) tightly inside its β -barrel, it is generally assumed to play a role in the transport of lipophilic molecules and in increasing their bioavailability [1,3]. β -LG has two tryptophan (Trp) residues: Trp19, located inside the calyx, has a very low fluorescence quantum yield presumably due to quenching by Arg 124; and Trp61, located at the mouth of the β -barrel and exposed to the solvent, is responsible for the protein fluorescence in the UV [4]. At the pH and protein concentration of milk, β -LG is present in the form of dimers interacting by hydrophobic forces and extensive hydrogen bonding involving a small β -strand not included in the β -barrel and the single long α -helix [4]. Trp 61 and Cys 121 are located at this interface and have been suggested to be involved in stabilization of the dimer [5]. The dimerization constant has been shown to depend strongly on pH and ionic strength and is reported to be $\sim 10^5 \text{ M}^{-1}$ at neutral pH and ionic strength of 0.1 M [6–8]. The monomer–dimer equilibrium is shifted towards the monomer by lowering the pH, increasing the temperature, or decreasing the ionic strength. Another important conformational change depen-

dent on the pH is the Tanford transition where the protonation of Glu 89 drives the movement of some of the loops connecting the β -strands towards the entrance to the hydrophobic calyx thereby closing it [9].

There is a large amount of literature reporting the binding of hydrophobic molecules to β -LG (see [1] for a recent review). Most ligands are shown to bind inside the hydrophobic calyx but there is also evidence for a secondary binding site located at the interface between the calyx exterior and the long α -helix.

In this report we have quantitatively evaluated the binding of a phospholipid derivative to β -LG and obtained some insight on the binding site through the dependence of binding on pH and on the monomer–dimer equilibrium of β -LG. The monomer–dimer equilibrium of β -LG was also quantitatively studied via changes in the fluorescence anisotropy of Trp.

Involvement of β -LG in the transfer of phospholipids between lipid assemblies was evaluated through the measurement of the rate constants for insertion/desorption into/from a lipid bilayer in the liquid-disordered phase [10,11]. To distinguish between transfer of the phospholipid through the aqueous phase or via interaction between the protein-bound phospholipid and the lipid bilayer, the rate constants obtained were compared with those for the transfer using bovine serum albumin (BSA). In previous work reported by our laboratory, the transfer of amphiphiles from BSA to lipid bilayers, and vice versa, was shown to occur through the aqueous phase and not via catalyzed transfer [12].

The association of β -LG with lipid bilayers has been rather extensively studied. There is evidence for interaction with negatively charged bilayers and bilayers made from sphingomyelin [13,14] with an effect on the thermal behavior of the lipid and on the secondary

* Corresponding author. Fax: +351 239 827703.

E-mail address: mmoreno@ci.uc.pt (M.J. Moreno).

structure of the protein. However, no effects on either the lipid or the protein have been observed for the case of phosphatidylcholines [13–17]. In the present work we report evidence for interaction between β -LG and phosphatidylcholine (POPC) bilayers which accelerate the transfer of lipids between lipid assemblies but have no significant effect on the CD spectrum of the protein.

2. Materials and methods

2.1. Materials

β -Lactoglobulins extracted from bovine milk, with minimum purity of 90%, were purchased from Sigma Aldrich, Sintra, Portugal (Form A, Cat # L7880 and form A+B, Cat # L0130). Bovine serum albumin (BSA) was essentially free of fatty acids and was purchased from PVL, Famões, Portugal (Cat # A0848). The fluorescent phospholipid derivative NBD- diC_{10}PE was synthesized as described earlier [18] by addition of NBD-chloride (Cat # 163260, Sigma Aldrich Sintra, Portugal) to di-decanoyl-phosphatidylethanolamine (diC_{10}PE), (Cat # 850700P, from Avanti Polar Lipids Inc., Alabaster, Alabama, USA) in chloroform solution at a molar proportion of 10 parts NBD-chloride to 9 parts diC_{10}PE in the presence of a slight excess of sodium carbonate as a base. The reaction mixture was allowed to stand for about 12 h at room temperature, with stirring and protected from light, after which the desired product was isolated and purified by preparative thin layer chromatography on Silica Gel 60 plates (Merck Portuguesa) using chloroform/methanol/acetic acid (80:20:1, vol.) as eluant. The phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-Dimyristoyl-*sn*-Glycerol-3-Phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl), Ammonium Salt (NBD-DMPE) were purchased from Avanti Polar Lipids Inc., Alabaster Alabama, USA (Cat # 850457 P and 810143, respectively). *N*-(6-tetramethylrhodaminethiocarbonyl)-1,2-di-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (TRITC-DHPE) and *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (dansyl DHPE) were purchased from Molecular Probes, Invitrogen, Portugal (Cat # T1391 and D57, respectively). All other chemicals were of analytical or higher purity.

2.2. Preparation of the solutions

Concentrations of NBD- diC_{10}PE (in methanol), TRITC-DHPE (in methanol), Dansyl-DHPE (in methanol) and β -LG or BSA (in aqueous solution) were determined by absorption spectrophotometry, assuming molar extinction coefficients of $21,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 463 nm for NBD- diC_{10}PE , $93,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 540 nm for TRITC-DHPE, $4500 \text{ M}^{-1} \text{ cm}^{-1}$ at 336 nm for Dansyl-DHPE, and $17,600 \text{ M}^{-1} \text{ cm}^{-1}$ for β -LG and 43,900 for BSA both at 278 nm.

Suspensions of NBD- diC_{10}PE in buffer or in protein solution (for equilibrium titration and transfer experiments) were prepared by squirting a solution of the lipid amphiphile in methanol (using a Hamilton syringe) into the desired volume of the solution, with simultaneous gentle vortexing for 1 min, taking care to ensure that the final methanol concentration was equal to 0.5%. These suspensions were always freshly prepared before use.

HEPES buffer (10 mM pH 7.4), used as aqueous medium, contained 0.15 M sodium chloride, 0.02% (m/v) sodium azide, and 1 mM EDTA, with or without β -mercaptoethanol at a final concentration of 1 mM.

Aqueous suspensions of lipids were prepared using the method described previously [19]. Briefly, a film of POPC, with or without fluorescent phospholipids, was prepared by evaporating the desired solution in an azeotropic chloroform/methanol mixture (87/13, v/v) and then left in a vacuum dessicator for at least 6 h at 23 °C. The solvent-free residue, was hydrated with 10 mM HEPES buffer, pH 7.4, with 0.15 M sodium chloride and 1 mM EDTA, at 23 °C, during at least 30 min with occasional vortexing. The amount of hydrating medium added was calculated to result in a final lipid concentration of 5 mM. The hydrated lipid was then extruded, using a minimum of 10 passes, through two stacked polycarbonate filters with a pore diameter of 0.1 μm (Cat # 110605 Nucleopore, purchased from VWR, Carnaxide, Portugal) [20], in an extruder from Lipex Biomembranes, Inc.

2.3. β -LG monomer-dimer equilibrium and binding of NBD- diC_{10}PE

Anisotropy of Trp fluorescence was used to study the β -LG monomer-dimer equilibrium. Protein solutions, ranging in concentration from 0.1 to 25 μM , were prepared some hours prior to measuring the anisotropy in order to let the system reach equilibrium. The excitation wavelength used was 292 nm and fluorescence emission was collected from 340 to 360 nm.

Equilibrium titration of a $2.5 \times 10^{-9} \text{ M}$ solution of NBD- diC_{10}PE was done by adding the amphiphile to various β -LG concentrations, ranging from 0.5 to 20 μM and measuring its fluorescence along time. In the cases where the fluorescence decreased over time an exponential curve was fitted to the data and the value extrapolated at $t=0$ was used in the titration curve. Higher concentrations of ligand were also tested (up to 10 nM) and the decrease in fluorescence over time was more significant, we have therefore routinely used the smaller concentration of ligand that conduces to a small error in the measured fluorescence, 2.5 nM.

2.4. Transfer of NBD- diC_{10}PE between β -LG, or BSA, and LUV prepared from POPC

Since the fluorescence quantum yield of NBD- diC_{10}PE bound by β -LG or incorporated into bilayer membranes of POPC is comparable, the transfer of NBD- diC_{10}PE between β -LG and POPC membranes presented as LUV was studied by following the increase in fluorescence of TRITC-DHPE at 575 nm, when excited at 450 nm, due to energy transfer from NBD- diC_{10}PE inserted in the LUV. The transfer of NBD- diC_{10}PE between BSA and POPC was followed both by the method above and also by the increase in the fluorescence intensity of NBD when inserted in the lipid bilayer as compared to bound to BSA as described previously [21]. For transfer from protein to LUV, different concentrations of LUV (containing only POPC or TRITC-DHPE:POPC 1:2000) were added to a 30 nM NBD- diC_{10}PE previously incubated with 40 μM β -LG. For the transfer from LUV to protein the process was similar but the LUV contained always NBD- diC_{10}PE at a ratio of 1 molecule per 5000 POPC molecules and some samples contained additionally TRITC-DHPE at a ratio of 1 to 2000 POPC molecules.

Absorption spectrophotometry was done using a Unicam UV530 absorption spectrophotometer and fluorescence and anisotropy measurements were done using a Cary Eclipse spectrofluorimeter with a thermostated multisample holder.

The experimental data obtained for anisotropy, equilibrium titration and transfer kinetics was analyzed with Solver from Microsoft Excel 2003.

2.5. Interaction of β -LG with POPC bilayers

The interaction between β -LG and POPC bilayers was studied *via* changes in the protein fluorescence intensity and Circular Dichroism spectra (using an Olis® DSM 20 CD spectrophotometer). Aqueous solutions of β -LG at concentrations of 2, 5 or 40 μM , with or without β -mercaptoethanol, were incubated with different concentrations of POPC (0 to 500 μM), as LUV, for 1 to 3 h at 25 °C. No effect of incubation time was observed for incubations up to 6 h.

The effect of β -LG on the exchange of the fluorescent phospholipid derivative, NBD-DMPE between POPC LUV was evaluated through the time dependence and the extent of energy transfer between NBD-DMPE and TRITC-DHPE, initially present in different LUV at a molar ratio of 1 fluorescent phospholipid for 200 POPC. TRITC-DHPE, with longer acyl chains, is expected to exchange at a much smaller rate [21], and this fluorescent phospholipids is usually considered a non-exchangeable probe [22]. Therefore, the observed increase in energy transfer can be attributed exclusively to the exchange of NBD-DMPE.

3. Results

3.1. Monomer-dimer equilibrium of β -LG

To characterize the monomer-dimer equilibrium of β -LG at the pH and ionic strength used (10 mM HEPES buffer, pH=7.4, with 0.15 M NaCl and 1 mM EDTA) we have studied the intrinsic fluorescence of the protein in the steady state. The fluorescence intensity was linearly dependent on the concentration of β -LG in the range 0.1 to 10 μM showing that Trp fluorescence quantum yield in the monomer and in

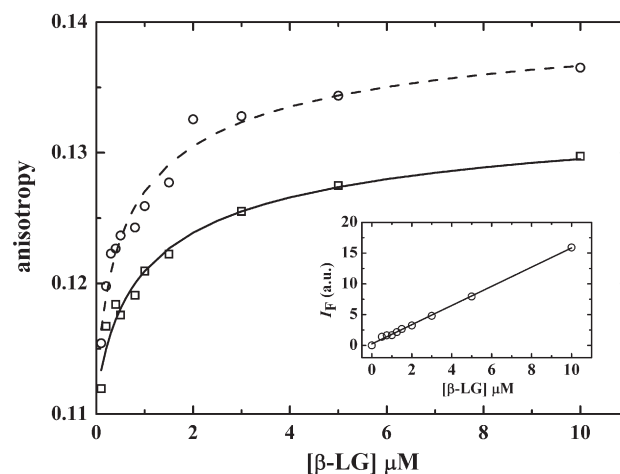


Fig. 1. Fluorescence anisotropy of β -LG as a function of its concentration in aqueous solutions with 10 mM HEPES, pH=7.4, 0.15 M NaCl (—, \square) and with 1 mM β -ME (---, \circ). The lines are the best fit of Eq. (1) with $K_D=5.3 \times 10^5 \text{ M}^{-1}$, $r_M=0.111$ and $r_D=0.136$ in the absence of β -ME, and $K_D=8.5 \times 10^5 \text{ M}^{-1}$, $r_M=0.112$ and $r_D=0.143$, with 1 μM β -ME. The inset shows the linear dependence of the fluorescence intensity with the concentration of β -LG.

the dimer are the same. Its fluorescence anisotropy is however dependent on the concentration of protein and allowed us to follow the monomer–dimer equilibria. The results obtained at 25 °C are shown in Fig. 1, both in the absence and presence of 1 mM β -mercaptoethanol (β -ME), and the dimerization constant was obtained from the best fit of Eq. (1);

$$\begin{aligned} r &= (2[D]r_D + [M]r_M)/[T] \\ [M] &= (-1 + \sqrt{1 + 8K_D[T]})/4K_D \\ [D] &= ([T] - [M])/2 \end{aligned} \quad (1)$$

where r_D and r_M are anisotropies of the monomer and dimer forms, $[M]$, $[D]$ and $[T]$ are the concentrations of monomer, dimer and total concentration of β -LG and K_D is the dimerization constant.

Most experiments performed in the absence of β -ME led to results that were not adequately fitted by Eq. (1) and that were dependent on the age of the sample after dilution to the desired concentration. The data shown in Fig. 1 was obtained after allowing the solutions to equilibrate for several hours after dilution. This behavior was interpreted in light of the possible involvement of Cys121 in the formation of disulfide bridges in dimer stabilization [5,23]. The formation of disulfide bridges in the dimer is controversial [4] but we have found a better reproducibility of the results obtained in the presence of 1 mM β -ME. Several studies have been performed where Cys121 was reacted with different groups and a stabilization of the monomer with a decrease in K_D was usually observed [24,25]. Those results are probably a consequence of the irreversible modification, with addition of bulky groups, of the β -LG surface involved in the dimer formation. In contrast, in our experimental setup β -ME interacts reversibly with Cys121 and we have found no significant effect on the dimerization equilibrium constant although the kinetics at which the equilibrium is attained may be accelerated, the value given for the dimerization constant is an average of several experiments performed both in the presence and absence of β -ME. In accordance with our results, the equilibrium was also shown to be unaffected by the presence of 1 mM dithiothreitol [26]. Some effects of β -ME on the fluorescence of the protein were however observed; a small increase in the fluorescence intensity (data not shown) and in the fluorescence anisotropy of both monomer and dimer, Fig. 1. The value obtained for K_D , $(7.0 \pm 1.5) \times 10^5 \text{ M}^{-1}$, is somewhat larger than the published values, at neutral pH and temperature around 20 °C, which range from 5×10^4 to $2 \times 10^5 \text{ M}^{-1}$ [6–8,26]. The difference may be due to our slightly basic pH or higher ionic strength. It should also be noted that we have used concentrations of β -LG down to 0.1 μM while most studies do not extended their protein concentration below 1 μM due to sensitivity of the method used. For the lower concentrations of β -LG, even the fluorescence anisotropy has a large uncertainty associated and the results shown in Fig. 1 could only be obtained when the stability of the equipment was optimal. Additionally, aggregation of β -LG to form larger aggregates has been reported [8] and, if those association steps are not well separated in terms of concentration, a different association constant is expected depending on the concentration range observed. In fact, the anisotropy of β -LG for solutions with higher concentrations (up to 20 μM) is higher than predicted by the best fit of the results shown in Fig. 1 indicating a continuous increase in the size of the aggregate.

3.2. Binding of NBD-diC₁₀PE to β -LG

The binding of the phospholipid derivative NBD-diC₁₀PE was followed through the increase in NBD fluorescence upon binding to β -LG. It was observed that the fluorescence of NBD-diC₁₀PE in aqueous solution was not stable but decreased over several minutes even at the very low concentration (2.5 nM) used in this work. The magnitude of the decrease was dependent on the concentration of NBD-diC₁₀PE and was attributed to aggregation. In the presence of high concentrations

of β -LG most of the NBD-diC₁₀PE is bound to the protein, its concentration in the aqueous phase is below the critical aggregation concentration (CAC), and the fluorescence intensity was stable over several hours. To correct for the effect of aggregation, the fluorescence of the ligand was followed over time and the amount of ligand bound in the absence of aggregate was obtained from the best fit of an exponential curve and extrapolation to zero time, Fig. 2 inset. Three independent experiments were performed and the average and standard deviation obtained are presented in Fig. 2. The binding constant was obtained from the best fit of

$$I_F = [\text{NBD-diC}_{10}\text{PE}]_T \frac{\Phi_A + \Phi_{AB}K_B[B]}{1 + K_B[B]} \quad (2)$$

where I_F is the measured fluorescence intensity, Φ_A and Φ_{AB} are the relative fluorescence quantum yields of NBD-diC₁₀PE in the aqueous solution and bound to the binding agent, B. Eq. (2) assumes one binding site per β -LG monomer and no effect of dimerization on the binding constant. The fit to the results obtained is quite good and the recovered binding constant, at 25 °C, was $(1.2 \pm 0.2) \times 10^6 \text{ M}^{-1}$. It should be noted that if the aggregation of NBD-diC₁₀PE was not taken into consideration, and the solutions were allowed to attain equilibrium, the apparent binding constant obtained would have been significantly smaller. The magnitude of this effect depends on the ratio of the total concentration used to the CAC. For the smaller concentration used, 2.5 nM, the apparent binding constant would be $8 \times 10^5 \text{ M}^{-1}$. With higher ligand concentrations the apparent binding constant is smaller but the binding constant obtained using the extrapolation to time zero was not significantly affected up to a total ligand concentration of 10 nM.

A reasonable estimate of the critical aggregation constant (CAC) of NBD-diC₁₀PE may be obtained from the time-dependence of the fluorescence of this probe in the presence of β -LG. When the protein concentration is high enough that the concentration of the free probe in aqueous solution is equal to or below its CAC, no time-dependent reduction in probe fluorescence will be observed since there is no aggregation of the free probe. We observed no time-dependent decrease in NBD-diC₁₀PE fluorescence when the β -LG concentration was equal to, or above, 2 μM . Given that $K_B = 1.2 \times 10^6 \text{ M}^{-1}$, this corresponds to a concentration of free NBD-diC₁₀PE in the aqueous phase of 0.7 nM, which is our estimate for the CAC of NBD-diC₁₀PE.

As was observed for the monomer–dimer equilibrium of β -LG, the binding was not affected by the presence of 1 mM β -ME except for a better reproducibility.

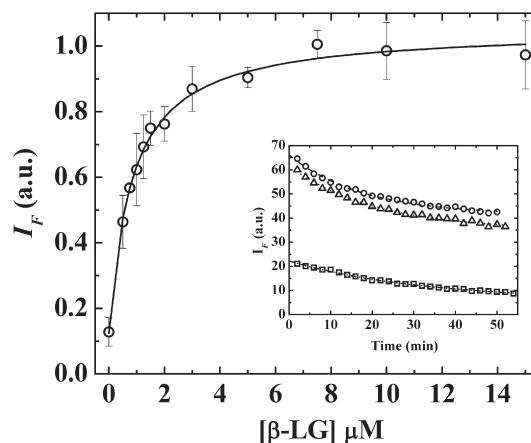


Fig. 2. Average values, and standard deviation, of the fluorescence intensity of 2.5 nM NBD-diC₁₀PE as a function of β -LG concentration for three independent experiments. For a β -LG concentration below 2 μM the fluorescence from NBD-diC₁₀PE decreased over time, as shown in the insert, and the intensity plotted is the extrapolation to $t=0$. The line is the best fit of Eq. (2) with $K_B = 1.2 \times 10^6 \text{ M}^{-1}$. The inset shows fluorescence of NBD-diC₁₀PE as a function of time for a total concentration of 2.5 nM and for different concentrations of β -LG; 0 (\square), 0.5 (Δ) and 1 μM (\circ). The line is the best fit of an exponential curve.

There are reports in the literature regarding two different binding sites on β -LG, one at the hydrophobic calyx and the other at the interface between the β -barrel and the long amphipathic α -helix [1,27]. Retinol, the most commonly studied ligand of β -LG, was shown to bind at the hydrophobic calyx [1] while the binding site of fatty acids has been controversial [1,27–29]. This protein undergoes a conformational transition centered at neutral pH, a so-called Tanford transition, where the entrance to the hydrophobic calyx is open at neutral and basic pH values and closed at acidic pH. This transition was used to evaluate the binding site for NBD-diC₁₀PE, Fig. 3. It was observed that lowering the pH to 5.5 decreased the ligand fluorescence to about half while an increase to pH 8.5 led to a small increase. This change in fluorescence could be due to a change in the ligand fluorescence quantum yield or to a change in the fraction of ligand bound. The observation of the slow decrease in fluorescence at low pH is a strong indication that it is the amount of ligand bound, and not its fluorescence quantum yield, that has changed. At pH 8.5, the high concentration of β -LG used (10 μ M) was enough to reduce the concentration of NBD-diC₁₀PE to near its critical aggregation constant ($[\text{diC}_{10}\text{-NBD}]_{\text{T}}=20$ nM, $[\text{diC}_{10}\text{-NBD}]_{\text{W}}\approx 1$ nM, $[\text{diC}_{10}\text{-NBD}]_{\text{Bound}}\approx 19$ nM) and the fluorescence is stable. When the pH is reduced to 5.5 the amount of ligand bound is reduced to about half leading to a concentration of ligand in the aqueous phase well above its CAC and it aggregates with a concomitant decrease in its fluorescence. This experiment was performed at different concentrations of β -LG and the results indicate a decrease of about an order of magnitude in the binding constant (data not shown). This decrease in the binding constant has been observed for other ligands that bind at the hydrophobic calyx [1,30,31] and is strong evidence for this to be the binding site for NBD-diC₁₀PE. Further evidence was obtained from the high fluorescence anisotropy of the ligand bound to β -LG, $r=0.17\pm 0.02$, which indicates tight binding and also from its high fluorescence intensity (comparable to that when inserted in a lipid bilayer) pointing to a very hydrophobic environment. The observation that the titration curve was well fitted by a single binding constant indicates that the binding is not affected by the monomer–dimer equilibrium. This is further evidence for the binding to the hydrophobic calyx because the properties of the secondary binding site, at the long α -helix, are expected to be affected by the monomer–dimer equilibrium due to its involvement in the stabilization of the dimer.

There are several reports in the literature for the binding of amphiphilic molecules to β -LG (see e.g. [1,32]). However, as far as we know, the binding of a two acyl chain phospholipid was never studied. In general, binding constants for amphiphiles reported in the literature often vary by several orders of magnitude for the same

ligand depending on the method used. We believe that this is due to the fact that in most, if not all, studies the ligand concentration is well above its CAC and ligand aggregation is not taken into account. Keeping that in mind, the binding constant found for lysoPC (mainly C16 and C18) to tear lipocalin (a protein structurally related to β -LG) [32] was similar to that found for NBD-diC₁₀PE in this work.

The equilibrium binding constant was measured as a function of temperature in the range 15 to 35 °C and this allowed us to resolve the Gibbs free energy into its enthalpic and entropic contributions. The binding was found to be essentially driven by entropy ($T\Delta S^0(25\text{ °C})=22\pm 5$ kJ/mol) with a small stabilizing enthalpic contribution ($\Delta H^0=-13\pm 5$ kJ/mol). This indicates that the hydrophobic effect is the main driving force for the interaction between the amphiphile and β -LG.

The binding to the commercially available mixture of β -LG forms A and B was also evaluated. The binding constant obtained was about half the value for β -LGA for temperatures between 15 and 35 °C. This result shows that the binding constant to form B is significantly smaller than to form A. The exact value cannot be calculated from the use of the mixture of forms because the fraction of each form is not specified. The two forms differ only by the replacement of Asp64 by Gly and Val118 by Ala in form B. The residue at position 64 is located near the calyx entrance [2,4] and the stronger binding of NBD-diC₁₀PE to form A may result from electrostatic interactions between the Asp residue and the ethanolamine group. Additionally, in crystals of form B, obtained from solutions with neutral pH, the calyx entrance was closed in contrast with the open calyx found for form A [2]. This may be another reason for the smaller binding constant and supports the identification of the hydrophobic calyx as the binding site for this amphiphile. Due to the smaller affinity for form B we have not proceeded further with the study of the mixture of the two forms nor have we further characterized the pure form B.

3.3. Transfer of NBD-diC₁₀PE between β -LG (or BSA) and lipid bilayers

The kinetics of NBD-diC₁₀PE transfer between β -LG and POPC bilayers is relevant in two ways: i) if the transfer is mediated by interactions between the protein and the lipid bilayer, the insertion/desorption may be catalyzed by the protein and this may be a physiologically important role for this protein; ii) if the transfer is mediated by the amphiphile in the aqueous phase, the kinetics are not affected by the protein except for the lower concentration of ligand in the aqueous phase due to binding to the protein. In the second case the protein may be used to obtain the rates of insertion/desorption of amphiphiles into/from lipid bilayers [33–36]. The kinetic scheme for transfer mediated by the ligand in the aqueous phase (ii) is presented in Eqs. (12,21,3)

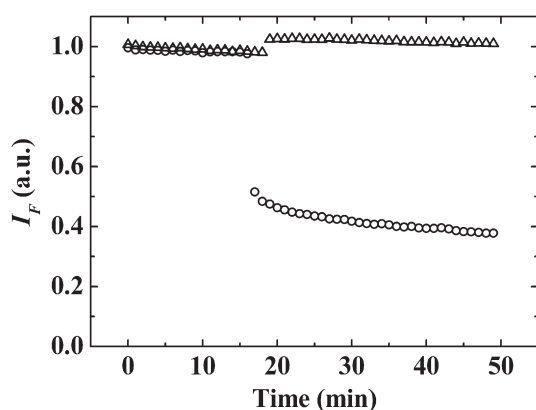
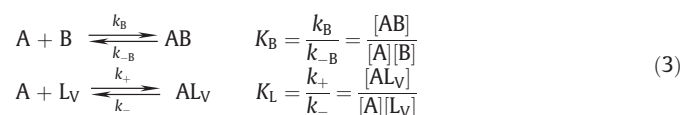


Fig. 3. Fluorescence intensity as a function of time for 10 μ M β -LG with 20 nM NBD-diC₁₀PE in aqueous solutions with 10 mM HEPES and 1 mM β -ME pH 7.4, in which the pH was modified to pH 8.5 (Δ) and pH 5.5 (\circ), by addition of NaOH and HCl, respectively.



where A, AB and AL_v is the amphiphile (ligand) in the aqueous phase, bound to the binding agent (B, protein) and inserted in the lipid bilayer of the large unilamellar vesicles (L_v).

If the transfer is mediated by the protein there are several possible kinetic schemes depending on whether the protein interacts only transiently with the LUVs or remains adsorbed or inserted in the lipid bilayer [37]. This situation may be distinguished from the case of transfer mediated by ligand in the aqueous phase, Eq. (3), based on the transfer rate observed and its dependence on the concentration of B and L_v [12]. We have previously shown that bovine serum albumin (BSA) does not interact with zwitterionic lipid bilayers and this protein has been used by us as a binding agent to avoid aggregation of the amphiphile and allow the characterization of the rate constants for its interaction with lipid bilayers [12,21,33–36].

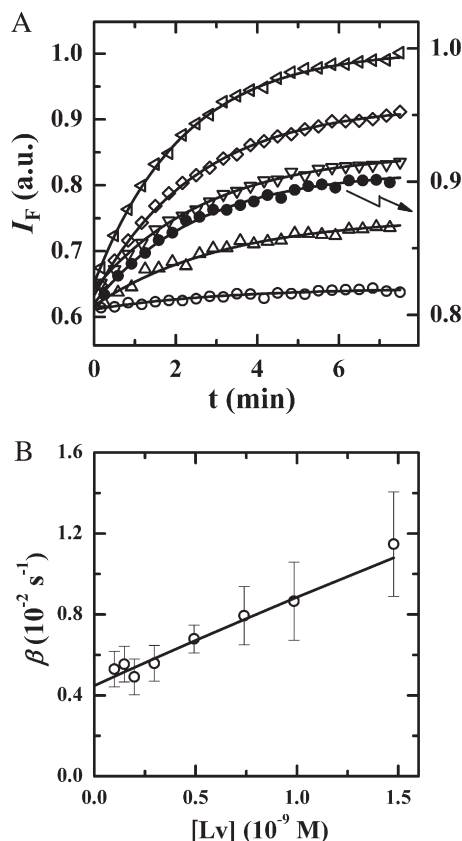


Fig. 4. Transfer of NBD-diC₁₀PE from BSA to LUVs prepared from POPC. Panel A – Time dependence of the increase in TRITC-DHPE fluorescence at 575 nm, due to energy transfer from NBD-diC₁₀PE being transferred to the LUV membrane from BSA. The LUVs contained 0.1 mol % TRITC-DHPE. $[Lv]$ was 0.098 (\circ), 0.30 (Δ), 0.49 (∇), 0.74 (\diamond) and 0.99 nM (\bullet). The increase in fluorescence of NBD-diC₁₀PE at 530 nm due to transfer to POPC LUVs at 0.49 nM is also shown (\bullet). The lines are the best fit with a mono-exponential equation. Panel B – Dependence of the transfer rate constant on the concentration of LUVs, average and standard deviation of 5 independent experiments. The line is the best fit of Eq. (4) with $k_+ = 9.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $k_- = 4.1 \times 10^{-3} \text{ s}^{-1}$ and $k_{-B} \geq 0.1 \text{ s}^{-1}$.

There are several reports in the literature where it is shown that β -LG does not interact with phosphatidylcholine bilayers [13,15,17]. We therefore expected that the transfer of amphiphile from β -LG to LUVs prepared from POPC could be described by Eq. (3). To obtain further support on the mechanism of transfer, we have compared the results obtained with β -LG with those obtained using BSA as the binding agent.

The results obtained for the transfer of NBD-diC₁₀PE from BSA to LUVs prepared from POPC are presented in Fig. 4. The dependence of the transfer rate, β , on the concentration of LUV was fitted with Eq. (4) which is obtained from the resolution of the differential equations describing the kinetic scheme (3) with the steady state approximation for the ligand in the aqueous phase [38].

$$\beta = \frac{k_+[Lv] + k_-(1 + K_B[B]_T)}{1 + K_B[B]_T + \frac{k_-}{k_{-B}}[Lv]} \quad (4)$$

The reverse transfer, from LUVs to BSA, has also been followed. If the mechanism of transfer is described by the kinetic scheme in Eq. (3), the transfer rate should be independent of the direction of net transfer. In fact, the two experiments led to the same values, within experimental error, and both have been used to calculate the average and standard deviation represented in Fig. 4 plot B.

From the best fit we obtain the insertion and desorption rate constants: $k_+ = 9.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $k_- = 4.1 \times 10^{-3} \text{ s}^{-1}$, the transfer rate showed a linear dependence on $[Lv]$ in the range observed which

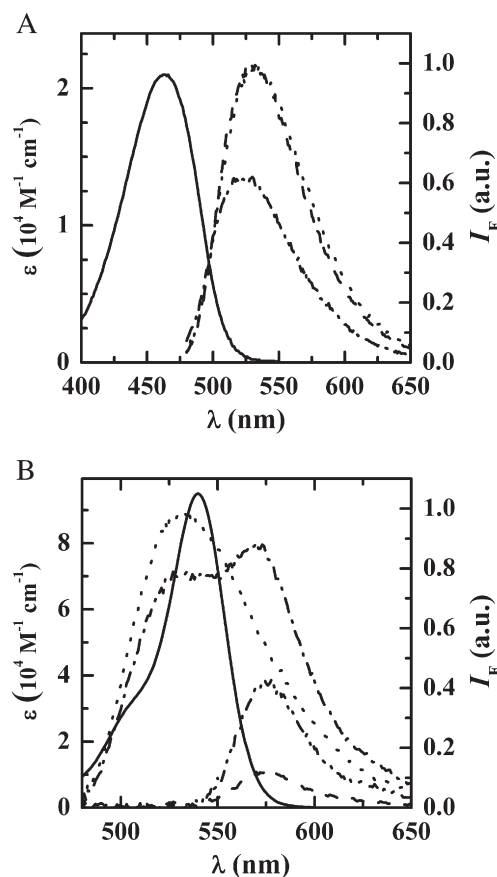


Fig. 5. Spectroscopic properties of the amphiphiles NBD-diC₁₀PE and TRITC-DHPE. For all fluorescence spectra the excitation was at 450 nm. Panel A – Absorption spectrum of NBD-diC₁₀PE in methanol (—); Fluorescence spectra of 30 nM NBD-diC₁₀PE in the presence of 30 μM BSA (---), 30 μM β -LG (---) or incorporated into POPC membranes (-.-). Panel B – Absorption spectrum of TRITC-DHPE in methanol (—); Fluorescence spectra of 30 nM NBD-diC₁₀PE in POPC LUVs (---), 30 nM TRITC-DHPE in POPC LUVs (---), and 30 nM NBD-diC₁₀PE + 30 nM TRITC-DHPE in POPC LUVs (-.-). The fluorescence from NBD was subtracted from the spectrum obtained with both fluorophores in the same LUV to obtain the fluorescence due to TRITC (---) and highlight the enhancement of its fluorescence due to energy transfer from NBD-diC₁₀PE. The fluorescence spectra have been normalized relative to that of NBD-diC₁₀PE bound to β -LG at 529 nm.

means that $k_+[Lv] \ll k_-$ and therefore only a lower limit for k_{-B} may be obtained, $k_{-B} \geq 0.1 \text{ s}^{-1}$. The equilibrium constant for the interaction between NBD-diC₁₀PE and LUVs prepared from pure POPC was independently measured and led to $K_L = 1 \times 10^{10} \text{ M}^{-1}$, which is consistent with the value calculated from the rate constants, $K_L = k_+/k_-$.

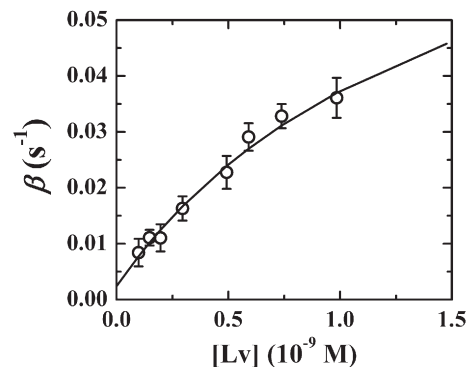


Fig. 6. Rate of transfer of NBD-diC₁₀PE from β -LG to POPC LUVs containing 0.1 mol % of TRITC-DHPE as a function of the LUV concentration (average and standard deviation of 4 independent experiments). The line is the best fit of Eq. (4) with $k_+ = 2.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, $k_- = 2.4 \times 10^{-3} \text{ s}^{-1}$ and $k_{-B} = 9.1 \times 10^{-2} \text{ s}^{-1}$.

The transfer of NBD-diC₁₀PE from β -LG to LUVs prepared from POPC could not be followed using the same methodology because the fluorescence intensity of the ligand is the same when bound to the protein and inserted in the lipid bilayer, Fig. 5 plot A. To follow the transfer we have prepared LUVs containing TRITC-DHPE (an efficient acceptor of energy in resonance transfer from the NBD excited state) and followed the enhancement of TRITC fluorescence when NBD-diC₁₀PE is inserted in the lipid bilayer, Fig. 5 plot B. To verify that this methodology is equivalent to the transfer between protein and LUVs prepared from pure POPC, in the absence of TRITC-DHPE, we have also followed the transfer between BSA and LUVs with TRITC-DHPE. The results were identical, within experimental error, and they have been included in the average and standard deviation shown in Fig. 4 plot B. In this approach, both the quenching of NBD and the enhancement of TRITC fluorescence can be followed. However, the enhancement of TRITC fluorescence shows a better signal to noise ratio and this was the methodology followed in most experiments. The results obtained for the transfer of NBD-diC₁₀PE between β -LG and LUVs of POPC with 0.1 mol% TRITC-DHPE are shown in Fig. 6. The rate of transfer was much faster than observed for BSA and when analyzed with Eq. (4) led to $k_+ = 2.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, $k_- = 2.4 \times 10^{-3} \text{ s}^{-1}$ and $k_{-B} = 9.1 \times 10^{-2} \text{ s}^{-1}$. The rate of desorption from the LUVs is similar to that obtained with BSA but the rate of insertion (and consequently the equilibrium constant) is about 40 times larger. In Eq. (4) it may be seen that the rate constant obtained for insertion is dependent on the binding constant to the protein (the larger the binding constant the smaller the fraction of ligand in the aqueous phase and therefore the slower the transfer). Because β -LG has a complex aggregation behavior and the concentration used in the transfer (40 μM) is higher than that used to calculate the binding constant (0.5 to 15 μM , Fig. 2) one should first evaluate whether the results obtained could be due to a smaller binding constant at the high protein concentration. To reconcile the data obtained with β -LG with the rate constants obtained with BSA as the binding agent, the equilibrium binding constant to β -LG needs to be $3.4 \times 10^4 \text{ M}^{-1}$. For this small binding constant, at a protein concentration of 40 μM and a total concentration of NBD-diC₁₀PE equal to 30 nM, the concentration of NBD-diC₁₀PE in the aqueous phase would be 13 nM. This concentration is well above its CAC (0.7 nM) and is incompatible with the finding that no aggregation is observed. We therefore conclude that the different transfer rate is not due to a small

binding constant but rather to a different transfer mechanism that involves interaction between the protein and the lipid bilayer.

3.4. Interaction between β -LG and POPC lipid bilayers

Although there is no evidence in the literature for interaction between β -LG and lipid bilayers prepared from phosphatidylcholines [13–17], the results obtained in the previous section indicate that a significant fraction of the protein must be in close proximity with the lipid bilayer of POPC. In the literature data, the interaction between β -LG and the lipid bilayers was assessed via changes in the structure of the protein (FTIR and/or CD) and/or in the thermotropic behavior of the lipid bilayer [13,14]. However, the unchanged tertiary structure of β -LG, or of order in the lipid bilayer, does not preclude adsorption of native protein to the surface of the bilayer. We have measured the effects of adding LUVs prepared from POPC on β -LG fluorescence and found evidence for interaction, Fig. 7. The fluorescence intensity is higher for small concentrations of LUV up to 1 nM (POPC concentration up to 100 μM) and decreases for higher concentrations of lipid. At 5 μM β -LG, the absorption by the protein at the wavelength used for excitation (292 nm) was 0.015 and the pseudo absorption due to light scattering from the LUV was 0.18 for the highest concentration of LUV used, being linearly dependent on LUV concentration. Therefore, the change observed in the fluorescence intensity is not expected to be due to inner filter effects but rather to a change in the environment of Trp. The interaction between β -LG and negatively charged lipid bilayers is well established [14,16,17]. Although phosphatidylcholines are neutral molecules, their bilayers possess a very high dipolar potential (positive in the bilayer interior [39,40]) due to orientation of charges and dipoles at the polar head group region, orientation of water in the hydration layer and orientation of the terminal methyl group of the acyl chains [39,41,42]. The native form of β -LG has a dipole moment of 730 Debye [43] and may therefore interact with both charged and/or dipolar surfaces.

To obtain some insight into the type of interaction that occurs between β -LG and POPC bilayers, we have measured the CD spectra of β -LG in the absence and presence of LUVs prepared from POPC, Fig. 7 plot B. No significant changes in the protein ellipticity were observed, in accordance with published results [16,17]. This points towards an electrostatic interaction between the protein and the surface of the

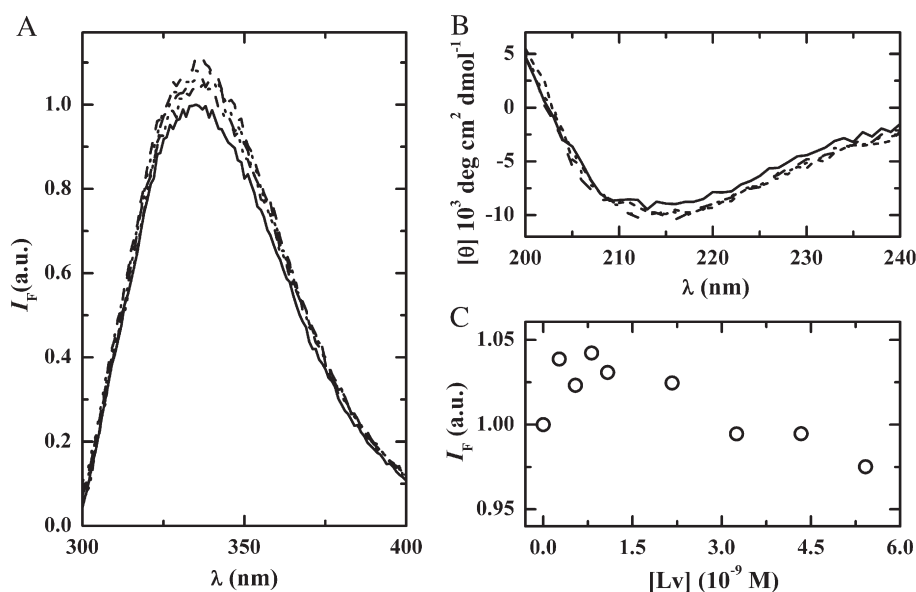


Fig. 7. Effect of POPC LUVs on the fluorescence spectra of 5 μM β -LG excited at 292 nm (Panel A) for different concentrations of POPC: 0 (—), 25 (— —), 50 (---), 400 (— · —) and 500 μM (·· ·). The fluorescence intensity at 335 nm, average of 4 independent experiments, is plotted as a function of POPC LUV concentration ($[LUV] = [\text{POPC}]/92300$) in Panel C. The far-UV CD spectra of β -LG is shown in Panel B for different concentrations of POPC: 0 (—), 75 (·· ·), 100 (— —) and 500 μM (---).

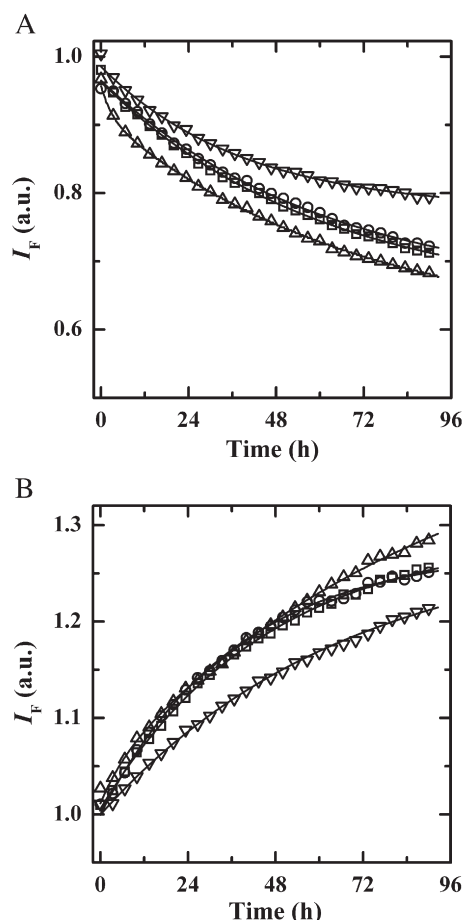


Fig. 8. Change in the fluorescence of NBD-DMPE at 530 nm (Panel A) or TRITC-DHPE at 575 nm (Panel B), when excited at 450 nm, due to equilibration of NBD-DMPE between POPC LUVs with 0.5 mol % NBD-DMPE and POPC LUVs with 0.5 mol % TRITC-DHPE both at a total lipid concentration of 25 μ M. The transfer occurred in the absence of protein (○), and in the presence of 5 μ M (□) or 40 μ M (Δ) β -LG or 40 μ M BSA (▽). The lines are the best fit of a mono-exponential with a rate constant of $(5 \pm 1) \times 10^{-6} \text{ s}^{-1}$ for all curves except in the presence of β -LG at 40 μ M where the line is the best fit of a bi-exponential with the rate constants $(5 \pm 1) \times 10^{-6} \text{ s}^{-1}$ and $(6 \pm 2) \times 10^{-5} \text{ s}^{-1}$.

lipid bilayer without changes in the 3-dimensional structure of the protein. The experiment was repeated with different total concentration of β -LG and the results were similar: i) a small increase in the fluorescence intensity up to a concentration of POPC of about 100 μ M followed by ii) a decrease in fluorescence intensity with the increase in the concentration of POPC. Although this behavior was systematic, we refrain from giving it a detailed interpretation due to its small magnitude. Energy transfer from Trp to a phospholipid with Dansyl as fluorescence acceptor (Dansyl-DHPE) was attempted. No energy transfer was observed at an acceptor density of up to 2 mol% in the POPC bilayer. The Förster radius for the Trp/Dansyl pair is about 20 Å [44], comparable to the radius of β -LG. Therefore, the absence of energy transfer is not in conflict with adsorption of the protein at the surface of the lipid bilayer. We are currently evaluating the possibility of chemical modification of β -LG with NBD or TRITC to proceed further with the elucidation of the interaction of β -LG with POPC bilayers using a donor/acceptor pair in energy transfer with a larger Förster radius.

3.5. Role of β -LG in the transfer of amphiphiles between lipid assemblies

In the previous section we have shown that β -LG interacts with POPC bilayers and this may be the reason why a fast transfer of the ligand NBD-diC₁₀PE from β -LG to LUVs was observed, Fig. 6. It is

important to know if this interaction enhances the rate of transfer of amphiphiles between lipid assemblies as this could be a possible physiological role for this protein. To test this hypothesis we have observed the rate of equilibration of NBD-diC₁₀PE between two populations of POPC LUVs, one initially with NBD-diC₁₀PE and the other initially with TRITC-DHPE. The rate of this process was however quite high even in the absence of protein, and was therefore not very sensitive. The characteristic time for exchange may be calculated from Eq. (4), considering the LUVs containing NBD-diC₁₀PE as the donor (instead of BSA) and the LUVs containing TRITC-DHPE as the acceptor and the corresponding rate and equilibrium constants, and is found to be about 4 min for a lipid concentration of 25 μ M ($[LUVs] = 0.3 \text{ nM}$) for both donor and acceptor LUVs. We have hence opted to follow the effect of the presence of β -LG in the rate of transfer of the slowly exchanging amphiphile NBD-DMPE ($k_- = 2.8 \times 10^{-5} \text{ s}^{-1}$, $k_+ = 2.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [21], $\tau_{\text{exchange}} \approx 10 \text{ h}$). As a control we have also measured the effect of BSA on the transfer rate and extent, the results are presented in Fig. 8. The rate of transfer observed was in reasonable agreement with that predicted from the published values for the rate constants for desorption and insertion of NBD-DMPE [21] for all curves except in the presence of β -LG at 40 μ M where a bi-exponential behavior was observed with the faster component being an order of magnitude faster than predicted. The smaller fluorescence variation observed in the presence of BSA, Fig. 8, is due to the efficient binding of NBD-DMPE to BSA [21]. Another observation is that in the presence of high concentrations of β -LG the extent of fluorescence variation is higher both from NBD-DMPE and from TRITC-DHPE. This finding, and the bi-exponential transfer observed, allows us to conclude that β -LG acts as a transfer protein increasing the rate of transfer of amphiphiles between lipid assemblies.

4. Discussion

The results shown clearly indicate that β -LG interacts efficiently with POPC lipid bilayers and that it increases the rate at which phospholipids exchange between different lipid aggregates. This finding is relevant for the biological function of the protein as it increases the rate of equilibration of amphiphilic molecules between the different lipid pools.

Our results provide some insight into the mechanism for an increased rate of amphiphile exchange in the presence of β -LG. This mechanism may well be applicable to the transfer and exchange of amphiphiles (lipids, sterols, xenobiotics, etc.) between membranes in a cell and between lipid assemblies (lipoproteins, membranes) and proteins in blood serum. In accordance with results reported by other laboratories, our results also indicate that the protein fluorescence and its CD spectrum are hardly altered in the presence of POPC membranes. However, we conclude that the protein is preferentially adsorbed to the POPC bilayer surface due to the interaction of its large dipole moment with the dipolar potential of the POPC bilayer. This adsorption, which is purely electrostatic in nature, does not involve a change in the protein structure or its insertion into the non-polar region of the bilayer. Under these conditions, a β -LG-associated amphiphile (NBD-diC₁₀PE in the present study) may exchange between the surface-adsorbed β -LG and the lipid bilayer without any changes in the intrinsic rate constants for amphiphile insertion/desorption. The finding that an efficient energy transfer between NBD-diC₁₀PE (initially bound to β -LG) and TRITC-DHPE (inserted in the POPC bilayers) is attained much faster than predicted via transfer of NBD-diC₁₀PE through the aqueous phase, is not in conflict with the previous statement because the Förster distance for the NBD/TRITC pair, 46 Å, is larger than the protein dimensions and the transfer of energy from NBD-diC₁₀PE to TRITC-DHPE in the lipid bilayer does not discriminate between a transfer from the NBD-diC₁₀PE bound to the surface-adsorbed β -LG and NBD-diC₁₀PE that may be incorporated into the bilayer.

The increased rate of exchange found may be understood if an encounter complex is considered as an intermediate in the insertion/desorption process [34]. An encounter complex is expected for any reaction involving two or more reactants occurring in condensed media. In the absence of interactions between the reactants, it is formed with a diffusion controlled rate and is broken down due to passive diffusion of the reactants away from each other which in aqueous media occurs with a characteristic time of some micro-seconds. During the lifetime of the encounter complex the amphiphile may insert in the lipid bilayer with a characteristic rate constant. If there are no interactions between the amphiphile and the surface of the lipid bilayer the local concentration of amphiphile (the one available for insertion) is equal to the concentration in the bulk aqueous media. If the protein interacts favorably with the surface of the lipid bilayer, its local concentration will be increased and so will be the local concentration of amphiphile leading to an increased rate of insertion even when the intrinsic rate constant is unchanged.

To describe quantitatively this mechanism of increased exchange, the rate constants for the interaction between the protein and the lipid bilayer, and the corresponding equilibrium constant, are required. The small effect observed on the intrinsic β -LG fluorescence due to the presence of POPC LUVs does not offer enough sensibility for such study. We are currently working on the chemical modification of β -LG with a fluorescent molecule to study the detailed kinetics of the interaction of β -LG with POPC LUVs using energy transfer.

Acknowledgements

This work was supported through research grants from the POCTI program of the Fundação para a Ciência e a Tecnologia (FCT) of the Portuguese Ministry for Higher Education and Scientific Research.

References

- [1] G. Kontopidis, C. Holt, L. Sawyer, Invited review: beta-lactoglobulin: binding properties, structure, and function, *J. Dairy Sci.* 87 (2004) 785–796.
- [2] B.Y. Qin, M.C. Bewley, L.K. Creamer, E.N. Baker, G.B. Jameson, Functional implications of structural differences between variants A and B of bovine beta-lactoglobulin, *Protein Sci.* 8 (1999) 75–83.
- [3] L. Sawyer, G. Kontopidis, The core lipocalin, bovine beta-lactoglobulin, *Biochim. Biophys. Acta, Prot. Struct. Mol. Enzymol.* 1482 (2000) 136–148.
- [4] S. Brownlow, J.H.M. Cabral, R. Cooper, D.R. Flower, S.J. Yewdall, I. Polikarpov, A.C.T. North, L. Sawyer, Bovine beta-lactoglobulin at 1.8 angstrom resolution – Still an enigmatic lipocalin, *Structure* 5 (1997) 481–495.
- [5] T.V. Burova, V.Y. Grinberg, V. Tran, T. Haertle, What may be bovine beta-lactoglobulin Cys121 good for? *Int. Dairy J.* 8 (1998) 83–86.
- [6] J.K. Zimmerma, G.H. Barlow, I.M. Klotz, Dissociation of beta-lactoglobulin near neutral pH, *Archives Of Biochemistry And Biophysics* 138 (1970) 101–109.
- [7] P. Aymard, D. Durand, T. Nicolai, The effect of temperature and ionic strength on the dimerisation of beta-lactoglobulin, *Int. J. Biol. Macromol.* 19 (1996) 213–221.
- [8] M. Verheul, J.S. Pedersen, S.P.F.M. Roefs, K.G. de Kruif, Association behavior of native beta-lactoglobulin, *Biopolymers* 49 (1999) 11–20.
- [9] B.Y. Qin, M.C. Bewley, L.K. Creamer, H.M. Baker, E.N. Baker, G.B. Jameson, Structural basis of the tanford transition of bovine beta-lactoglobulin, *Biochemistry* 37 (1998) 14014–14023.
- [10] J.H. Ipsen, G. Karlstrom, O.G. Mouritsen, H. Wennerstrom, M.J. Zuckermann, Phase-equilibria in the phosphatidylcholine–cholesterol system, *Biochim. Biophys. Acta* 905 (1987) 162–172.
- [11] K. Simons, W.L.C. Vaz, Model systems, lipid rafts, and cell membranes, *Ann. Rev. Biophys. Biomol. Struct.* 33 (2004) 269–295.
- [12] M.S.C. Abreu, L.M.B.B. Estronca, M.J. Moreno, W.L.C. Vaz, Binding of a fluorescent lipid amphiphile to albumin and its transfer to lipid bilayer membranes, *Biophys. J.* 84 (2003) 386–399.
- [13] T. Lefevre, M. Subirade, Structural and interaction properties of beta-lactoglobulin as studied by FTIR spectroscopy, *Int. J. Food Sci. Technol.* 34 (1999) 419–428.
- [14] T. Lefevre, M. Subirade, Interaction of beta-lactoglobulin with phospholipid bilayers: a molecular level elucidation as revealed by infrared spectroscopy, *Int. J. Biol. Macromol.* 28 (2000) 59–67.
- [15] D.G. Cornell, Lipid–protein interactions in monolayers – egg-yolk phosphatidic acid and beta-lactoglobulin, *J. Colloid Interface Sc.* 88 (1982) 536–545.
- [16] X. Zhang, N. Ge, T.A. Keiderling, Electrostatic and hydrophobic interactions governing the interaction and binding of β -lactoglobulin to membranes, *Biochemistry* 46 (2007) 5252–5260.
- [17] X.Q. Zhang, T.A. Keiderling, Lipid-induced conformational transitions of beta-lactoglobulin, *Biochemistry* 45 (2006) 8444–8452.
- [18] W.L.C. Vaz, D. Hallmann, Experimental-evidence against the applicability of the saffman–delbruck model to the translational diffusion of lipids in phosphatidylcholine bilayer-membranes, *Febs Lett.* 152 (1983) 287–290.
- [19] M.J. Moreno, L.M.B.B. Estronca, W.L.C. Vaz, Translocation of phospholipids and dithionite permeability in liquid-ordered and liquid-disordered membranes, *Biophys. J.* 91 (2006) 873–881.
- [20] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure – characterization of size distribution, trapped volume and ability to maintain a membrane-potential, *Biochim. Biophys. Acta* 812 (1985) 55–65.
- [21] M.S.C. Abreu, M.J. Moreno, W.L.C. Vaz, Kinetics and thermodynamics of association of a phospholipid derivative with lipid bilayers in liquid-disordered and liquid-ordered phases, *Biophys. J.* 87 (2004) 353–365.
- [22] R.E. Pagano, O.C. Martin, A.J. Schroit, D.K. Struck, Formation of asymmetric phospholipid-membranes via spontaneous transfer of fluorescent lipid analogs between vesicle populations, *Biochemistry* 20 (1981) 4920–4927.
- [23] S. Bouhallab, G. Henry, F. Caussin, T. Croguennec, J. Fauquant, D. Molle, Copper-catalyzed formation of disulfide-linked dimer of bovine beta-lactoglobulin, *Lait* 84 (2004) 517–525.
- [24] T.V. Burova, Y. Choiset, V. Tran, T. Haertle, Role of free Cys121 in stabilization of bovine beta-lactoglobulin B, *Protein Eng.* 11 (1998) 1065–1073.
- [25] R.K.O. Apenten, D. Galani, Protein stability function relations: native beta-lactoglobulin sulphhydryl–disulphide exchange with PDS, *J. Sci. Food Agric.* 80 (2000) 447–452.
- [26] M.J. Kelly, F.J. Reithel, Thermodynamic analysis of monomer–dimer association of beta-lactoglobulin-A at isoelectric point, *Biochemistry* 10 (1971) 2639–2644.
- [27] M. Narayan, L.J. Berliner, Fatty acids and retinoids bind independently and simultaneously to beta-lactoglobulin, *Biochemistry* 36 (1997) 1906–1911.
- [28] P. Puyol, M.D. Perez, J.M. Ena, M. Calvo, Interaction of bovine beta-lactoglobulin and other bovine and human whey proteins with retinol and fatty-acids, *Agric. Biol. Chem.* 55 (1991) 2515–2520.
- [29] B.Y. Qin, L.K. Creamer, E.N. Baker, G.B. Jameson, 12-Bromododecanoic acid binds inside the calyx of bovine beta-lactoglobulin, *Febs Lett.* 438 (1998) 272–278.
- [30] M. Collini, L. D'Alfonso, H. Molinari, L. Ragona, M. Catalano, G. Baldini, Competitive binding of fatty acids and the fluorescent probe 1-8-anilinonaphthalene sulfonate to bovine beta-lactoglobulin, *Protein Sci.* 12 (2003) 1596–1603.
- [31] Q.W. Wang, J.C. Allen, H.E. Swaisgood, Binding of retinoids to beta-lactoglobulin isolated by bioselective adsorption, *J. Dairy Sci.* 80 (1997) 1047–1053.
- [32] O.K. Gasyimov, A.R. Abduragimov, T.N. Yusifov, B.J. Glasgow, Binding studies of tear lipocalin: the role of the conserved tryptophan in maintaining structure, stability and ligand affinity, *Biochim. Biophys. Acta, Prot. Struct. Mol. Enzymol.* 1433 (1999) 307–320.
- [33] L.M.B.B. Estronca, M.J. Moreno, W.L.C. Vaz, Kinetics and thermodynamics of the association of dehydroergosterol with membranes, *Biophys. J.* 93 (2007) 4244–4253.
- [34] L.M.B.B. Estronca, M.J. Moreno, J.A.N. Laranjinha, L.M. Almeida, W.L.C. Vaz, Kinetics and thermodynamics of lipid amphiphile exchange between lipoproteins and albumin in serum, *Biophys. J.* 88 (2005) 557–565.
- [35] J.L. Sampaio, M.J. Moreno, W.L.C. Vaz, Kinetics and thermodynamics of association of a fluorescent lysophospholipid derivative with lipid bilayers in liquid-ordered and liquid-disordered phases, *Biophys. J.* 88 (2005) 4064–4071.
- [36] W.L.C. Vaz, E. Melo, Fluorescence spectroscopic studies on phase heterogeneity in lipid bilayer membranes, *J. Fluoresc.* 11 (2001) 255–271.
- [37] L.J. Falomir-Lockhart, L. Laborde, P.C. Kahn, J. Storch, B. Corsico, Protein-membrane interaction and fatty acid transfer from intestinal fatty acid-binding protein to membranes – Support for a multistep process, *J. Biol. Chem.* 281 (2006) 13979–13989.
- [38] J.I. Steinfeld, J.S. Francisco, W.L. Hase, Chemical Kinetics and Dynamics, Prentice-Hall, Inc., Upper Saddle River, NJ, 1999 07458.
- [39] G. Ceve, Membrane electrostatics, *Biochim. Biophys. Acta* 1031 (1990) 311–382.
- [40] H. Brockman, Dipole potential of lipid-membranes, *Chemistry And Physics Of Lipids* 73 (1994) 57–79.
- [41] S.J. Marrink, D.P. Tieleman, A.R. vanBuuren, H.J.C. Berendsen, Membranes and water: an interesting relationship, *Faraday Discuss.* (1996) 191–201.
- [42] R.J. Mashl, H.L. Scott, S. Subramaniam, E. Jakobsson, Molecular simulation of dioleoylphosphatidylcholine lipid bilayers at differing levels of hydration, *Biophys. J.* 81 (2001) 3005–3015.
- [43] X. Liu, L. Shang, X. Jiang, S. Dong, E. Wang, Conformational changes of [beta]-lactoglobulin induced by anionic phospholipid, *Biophys. Chem.* 121 (2006) 218–223.
- [44] W.L.C. Vaz, K. Kaufmann, A. Nicksch, Use of energy-transfer to assay association of proteins with lipid-membranes, *Anal. Biochem.* 83 (1977) 385–393.